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Mechanisms of Localization of Symbiont-produced Natural Products in Host Colony

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MECHANISMS OF LOCALIZATION OF SYMBIONT-PRODUCED NATURAL PRODUCTS IN HOST COLONY

by

FAHMINA AKHTER

Under the Direction of Dr. Nicole B. Lopanik

ABSTRACT

The relationship between the bryozoan *Bugula neritina* and its symbiont "*Candidatus* Endobugula sertula" is a specific yet complex model for defensive symbiosis where the host larvae obtain chemical protection from predation by symbiont-produced bryostatin. The symbiotic bacteria are located in both larval and adult tissues of the host. However, the bryostatins levels are higher in larvae and in adult zooids with ovicells where the larvae are brooded, compared to adult zooids without ovicells. In this study, symbiont cell density and bryostatin biosynthetic gene expression were quantified in host tissues, using TaqMan probe based Q-PCR, to investigate the mechanisms for the higher bryostatin content in larvae. The results showed that there are no significant differences of the symbiont cells in three tissues and the bryostatin gene expression is not different in ovicell-bearing zooids as compare to ovicell-free zooids suggesting that the host plays a role in transferring bryostatin to larvae.

INDEX WORDS: *Bugula neritina*, Symbiotic bacteria, Bryostatin, TaqMan probe, Q-PCR

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by

FAHMINA AKHTER

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Master of Science

in the College of Arts and Sciences

Georgia State University

2013

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Fahmina Akhter
2013

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PRODUCTS IN HOST COLONY

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Georgia State University

May 2013

DEDICATION

To my parents

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1 INTRODUCTION

Bugula neritina is a sessile, colonial invertebrate marine bryozoan usually found in fouling communities on dock sides, boat hulls, buoys and rocks (Gordon & Mawatari, 1992). *B. neritina* colonies are found in temperate and tropical coastal region around the world (Davidson & Haygood, 1999, McGovern & Hellberg, 2003, Mackie, *et al.*, 2006). Adult colonies consist of many individual zooids (Ryland, 1970) that are connected to each other by funicular cords, a tubular system within the colony (Woollacott & Zimmer, 1975, Sharp, *et al.*, 2007). Some zooids in a colony produce larval brood chambers called ovicells; these are often found on the upper central part of the colony, while the younger zooids at the tips of the colony are typically not reproductive (Woollacott & Zimmer, 1972). The funicular cords play an important role in transmitting nutrients within the colony and to the developing larvae (Woollacott & Zimmer, 1975). *B. neritina* harbors an uncultivated symbiotic γ -Proteobacterium "*Candidatus* Endobugula sertula" in both larval and adult stages (Woollacott & Zimmer, 1975, Woollacott, 1981, Haygood & Davidson, 1997). In a *B. neritina* larva, the bacteria are found within a globular invagination known as the pallial sinus located on the aboral side (Woollacott, 1981, Haygood & Davidson, 1997). In adult *B. neritina* zooids, the symbiotic bacterium is found in funicular cords (Woollacott & Zimmer, 1975, Sharp, *et al.*, 2007). The symbiotic bacterium "*E. sertula*" has not been found in the surrounding seawater column suggesting that it is transmitted vertically between *B. neritina* generations (Haygood, *et al.*, 1999). Another piece of evidence that suggests vertical transmission of the symbiont is that the symbiotic bacteria are internalized along with the larval pallial cells during the metamorphosis of *B. neritina* larvae (Reed & Woollacott, 1983).

Furthermore, occurrence of a microbial symbiont in host reproductive structures is indicative of vertical transmission (Cary & Giovannoni, 1993, Wilkinson, *et al.*, 2003).

The symbiont "*E. sertula*" is thought to be involved in the biosynthesis of bryostatins (Davidson, *et al.*, 2001, Lopanik, *et al.*, 2004a). Bryostatins are complex macrolactone polyketides, which are often biosynthesized by modular type I polyketide synthases (Pettit, 1991, Fischbach & Walsh, 2006). They have been shown to provide chemical protection to the soft, nutritious larvae of the host *B. neritina* from predators such as fish by making the larvae unpalatable (Lindquist & Hay, 1996, Lopanik, *et al.*, 2004b). Results from several studies have demonstrated that "*E. sertula*" is likely responsible for production of bryostatin. Antibiotic treatment of *B. neritina* larvae cures "*E. sertula*" resulting in the next generation without bryostatins. Further, extracts from aposymbiotic larvae were significantly more palatable than extracts from symbiotic larvae, thus demonstrating the ecological relevance of the bryostatins (Lopanik, *et al.*, 2004a). To date, 20 different bryostatins have been identified from *B. neritina* (Pettit, 1996, Davidson & Haygood, 1999, Lopanik, *et al.*, 2004a). It has been reported that bryostatin 1 has therapeutic potential in the treatment of cancers such as leukemia, lymphomas, melanomas and solid tumors (Mutter & Wills, 2000, Blackhall, *et al.*, 2001, Hayun, *et al.*, 2007, Ku, *et al.*, 2008, Lam, *et al.*, 2010), as well as Alzheimer's disease, a neurological disorder (Etcheberrigaray, *et al.*, 2004, Sun & Alkon, 2005). Adequate supply of bryostatin is limited due to inability to culture the symbiont bacterium in the laboratory. Moreover, bryostatins are found in very low yield in the adult *B. neritina* colonies. For instance, bryostatin 1 yield was 18 g from approximately 13,000 kg wet weight of *B. neritina* (Schaufelberger, *et al.*, 1991). Due to the ecological role and therapeutic potential of bryostatins, research on the interactions between the host and symbiont and production of bryostatin by the symbiont has gained interest.

Although "*E. sertula*" has yet to be cultured, the putative *bry* gene cluster was sequenced from a metagenomic library of *B. neritina*. The ~80 kbp bryostatin biosynthetic gene cluster (*bry*) that putatively encodes the bryostatin biosynthetic enzyme was sequenced from two distinct *B. neritina* sibling species: Deep and Shallow species (Hildebrand, *et al.*, 2004, Sudek, *et al.*, 2007) (Fig. 1). The Deep species is frequently found on the West coast of United States while the Shallow species is found on both West and East coast (Davidson & Haygood, 1999, McGovern & Hellberg, 2003). The *bry* gene cluster consists of five large modular genes (*bryA-D* and X) that encode polyketide synthases (PKS), and a four-gene cassette (*bryP-S*) that codes for two acyltransferase (AT) domains and other accessory function genes that are thought to be involved in bryostatin biosynthesis (Sudek, *et al.*, 2007). PKSs catalyze the synthesis of polyketides in a stepwise manner via multi-domain modules that elongate and modify the growing polyketide chain. The core domains, the acyl-transferase (AT), β -ketoacyl synthase (KS), and acyl carrier protein (ACP), are responsible for the elongation of the nascent polyketide chain by two carbons. The accessory domains, the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) reduce the β -carbonyl to various degrees resulting in a wide array of polyketide molecules. An acyl-CoA precursor (malonyl-CoA) acts as the extender unit selected by the AT domain and is added to the growing polyketide chain by condensation catalyzed by the KS domain (Hopwood & Sherman, 1990, Donadio, *et al.*, 1991, Fischbach & Walsh, 2006).

It has been shown that there is variation in the concentration of bryostatin in different life stages of *B. neritina*. The bryostatin levels are higher in larvae and in ovicell-bearing zooids than that in ovicell-free zooids. For example, bryostatin 10 levels in adult ovicell-bearing and ovicell-free tissue extracts were 78.4% and 91.5% lower than the level in larval extracts respectively (Lopanik, *et al.*, 2006). The higher concentration of bryostatins in ovicell-bearing zooids is

thought to be the result of the presence of larvae, which have very high levels of bryostatins thought to chemically protect them from predators (Lopanik, *et al.*, 2004b, Lopanik, *et al.*, 2006). Sharp and coworkers (2007) investigated the localization of bryostatin and symbiont bacteria during *B. neritina* life stages using Protein Kinase C (PKC) based detection method and fluorescent *in situ* hybridization (FISH) respectively. The presence of bryostatin was observed on the external surface of larvae while the symbiont was detected in the larval pallial sinus demonstrating that the location of bryostatins is not necessarily confined to the symbiont cells, and suggesting that bryostatins are loaded on the outer surface of larvae (Sharp, *et al.*, 2007). In the ovicell, bryostatin signal was identified near the base where the ovicell is attached to the feeding zooid via funicular cord, and surrounding the embryonic chamber. Symbiont bacteria were located in the funicular cord as well as in the ovicell base close to the bryostatin signal, suggesting that the bryostatins and the symbiont bacteria are transferred from the ovicell to the developing embryo via the funicular cords (Sharp, *et al.*, 2007).

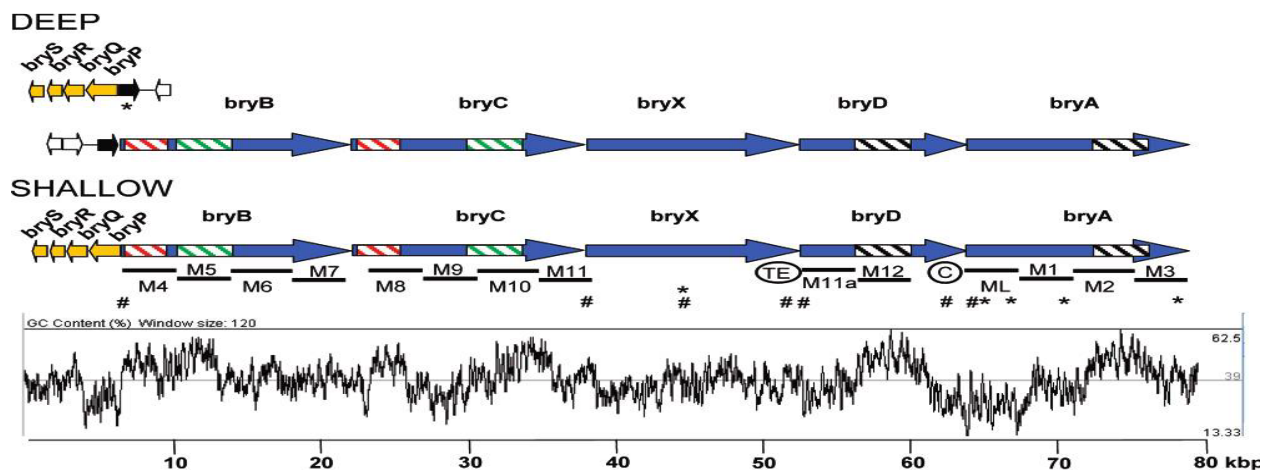


Figure 1: The proposed bry cluster in Deep and Shallow “*E. sertula*” involved in bryostatin biosynthesis (Sudek, *et al.*, 2007).

1.1 Purpose of the study

Marine invertebrates such as bryozoans, sponges, tunicates, and cnidarians often harbor symbiotic bacteria that are involved in the production of therapeutically important secondary metabolites (Dunlap, *et al.*, 2007). It is intriguing that most of these metabolites are localized to the host tissues and not bound to the symbiont cells (Salomon & Faulkner, 2002). It has been reported that the bioactive natural products, the patellamides, are produced by a cyanobacterial symbiont. While higher concentrations of patellmides occur in the zooid and tunic portion of the tunicate host *Lissoclinum patella*, the symbiont resides in the cloacal cavity of the host, suggesting that the patellamides are translocated to a region of the animal other than the site of biosynthesis (Salomon & Faulkner, 2002, Donia, *et al.*, 2006). Esquenazi and coworkers (2008) analyzed the distribution of therapeutically important metabolites produced by the cyanobacterium *Oscillatoria spongelliae* within the tissues of sponge *Dysidea herbacea*. The metabolites were differentially distributed at the pinacoderm tissue and at the edges of ostia, whereas even distribution of metabolites was observed within the mesohyl tissues implying the existence of chemically different environments within sponge tissues (Esquenazi, *et al.*, 2008). However, the mechanisms of the distribution of symbiont-produced secondary metabolites within host tissues are unknown.

The variation in bryostatin levels in different life stages of *B. neritina* has been reported in previous studies (Lopanik, *et al.*, 2004a, Lopanik, *et al.*, 2006, Sharp, *et al.*, 2007) and suggests some sort of coordination between host and symbiont, although, regulatory factors involved in bryostatin biosynthesis are yet to be determined. It is not known if the increase in bryostatin content in the ovicells and in larvae is due to an increase in the quantity of “*Candidatus* Endobugula sertula” cells, resulting in upregulation of bryostatin biosynthetic

genes, or an intermediary host factor is used to upregulate bryostatin biosynthesis by transmitting the environmental signal to the bacterial symbiont (Trindade-Silva, *et al.*, 2010). In this study, it is proposed that the higher concentrations of bryostatins in developing larvae may be the consequence of a greater number of symbiont cells in zooids that are brooding larvae; alternatively, bryostatin biosynthetic gene expression may be upregulated by symbionts in the ovicell-bearing zooids. Finally, it is possible that symbiont cell density as well as bryostatin production may occur uniformly throughout the colony and bryostatin transport occurs to the developing embryo via funicular cords during embryogenesis.

The first aim of this study is to quantify the symbiotic bacteria present in adult ovicell-bearing zooids, ovicell-free zooids, and larvae of *B. neritina* in order to determine if some tissue types have higher densities of symbiont cells than others, which would suggest translocation within the host colony. In this study, the number of symbiont cells normalized to the number of host cell was measured to identify symbiont cell density in each tissue. The quantity of symbiont cells was assessed using the 16S rRNA gene copy number as a proxy for cell number, whereas host cells were quantified using the 18S rRNA gene. Copy number of both genes in genomic DNA from each tissue type was calculated using the absolute quantification method and a standard curve from serial dilutions of PCR amplicons. The second aim of this research study is to quantify bryostatin biosynthetic gene expression to assess if the *bry* genes are up- or down-regulated in these three types of tissue indicating differential gene regulation. To account for possible *bry* gene differential expression in the symbiont, relative expression of two bryostatin biosynthetic genes (*bryA* and *bryB*) was measured using 16S rRNA gene expression as a baseline (Edwards & Saunders, 2001, Fey, *et al.*, 2004, Stevenson & Weimer, 2005). The relative quantification method (Klein, 2002) was applied to quantify bryostatin biosynthetic gene

expression, as it is usually used to compare the level of gene expression in different samples. In this method, the relative concentration of the bryostatin biosynthetic gene in experimental sample (ovicell-bearing and larvae tissue) was measured by comparing to a calibrator or control sample (ovicell-free tissue), which contains both the bryostatin biosynthetic gene and the reference gene. The ratio of target gene and reference gene in experimental sample was standardized by the ratio of target and reference gene in a control sample (Lee, *et al.*, 2008). The up- or down-regulation of the target gene in a sample relative to the control sample is expressed as fold-changes and determined by the differences in Ct value between the experimental sample and control sample. The Q-PCR experiments with relative quantification method allowed us to test the hypothesis that bryostatin biosynthetic gene expression is possibly upregulated in ovicell-bearing tissue of *B. neritina*. The results of this study allow us to identify the mechanisms for the difference in bryostatin content within the host colony.

2 MATERIALS AND METHODS

2.1 Collection of *B. neritina* adult tissues and larvae

B. neritina colonies were collected from floating docks in Morehead City and Beaufort, North Carolina. After collection, the *B. neritina* colonies were maintained in flowing seawater tables at the wet laboratory facilities of University of North Carolina at Chapel Hill's Institute of Marine Sciences. The colonies were covered with black plastic bags to ensure the maintenance of dark cycle for approximately 18 hours. *B. neritina* colonies release larvae during the day (Ryland, 1974, Wendt, 2000). For the collection of larvae, *B. neritina* colonies were placed in 4-liter glass jars filled with seawater and exposed to sunlight in the morning to induce the release of larvae. As the larvae aggregated toward the edge of the jar, a wide-tip glass pipette was used

to collect the larvae and placed into ice-cold seawater. Three hundred larvae (N=4) were collected into a 1.5 ml Eppendorf tube and the seawater was removed from the tubes. For the collection of ovicell-free and ovicell-bearing tissues (Fig. 2A & 2B), adult branches of *B. neritina* colony were dissected using a scalpel under a dissecting microscope and a total of 300 of ovicell-bearing and ovicell-free zooids were placed in a 1.5 ml Eppendorf tube (N=4 of each tissue type). The samples were preserved immediately in 1 ml of Trizol reagent (Invitrogen) for subsequent DNA and RNA extractions and stored at -80°C.

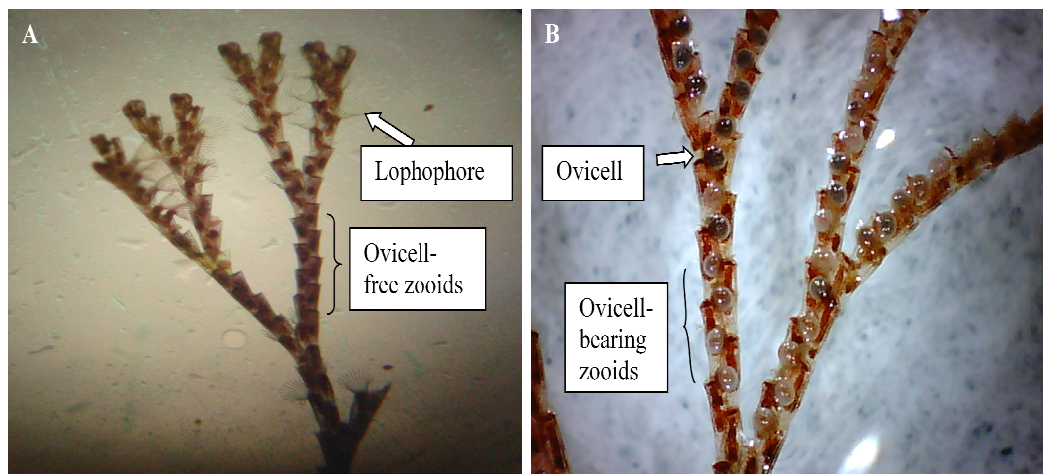


Figure 2: Adult zooids of *B. neritina*. A: ovicell-free zooids, white arrow (solid) is showing lophophore. B: ovicell-bearing zooids (black bracket), white arrow (solid) is showing ovicell (photo by Michelle Ventura).

2.2 Experimental approach

To quantify symbiont bacteria and bryostatin gene expression in each tissue type, TaqMan® probe based real-time Q-PCR was used in this study. Several studies have reported the use of TaqMan® probe based real-time PCR technique to quantify total bacteria in a variety of environments (Nogva, *et al.*, 2000, Suzuki, *et al.*, 2000, Ge, *et al.*, 2001, Hein, *et al.*, 2001, Bach, *et al.*, 2002, Nadkarni, *et al.*, 2002, Malinen, *et al.*, 2003). In real-time PCR, the change in fluorescence is observed and quantified during the exponential phase of PCR amplification (Suzuki & Giovannoni, 1996, Malinen, *et al.*, 2003). The TaqMan® probe based Q-PCR assay,

also known as 5'-nuclease assay (Holland, *et al.*, 1991), uses a dual-labeled fluorescent hybridization probe which specifically binds within target region to be amplified. The probe has a reporter fluorescent dye attached to the 5'-end and a quencher dye at 3'-end. The fluorescence of the reporter dye is quenched by the quencher dye using a FRET (Fluorescence Resonance Energy Transfer) quenching mechanism. The 5'-3' exonuclease activity of Taq DNA polymerase cleaves the probe during primer extension resulting in the release of fluorescence from the free reporter dye (Heid, *et al.*, 1996). TaqMan® assays are more sensitive and specific in contrast to SYBR green based real-time Q-PCR because the intercalating fluorescent SYBR Green dye binds to any double-stranded DNA and not specifically to target DNA.

2.3 Primers and probes design

Nucleotide sequences for symbiont 16S rRNA gene were obtained from the NCBI database (GenBank accession number AF06606, strain BnSP) and aligned with 16S rRNA sequences of the symbiont "*Endobugula glebosa*" of *B. simplex* (AY532642), the symbiont of *B. pacifica* (AY633929), symbiont of *B. turbinata* (AY633930), phylogenetically related symbiont *Teredinibacter turnerae* (AY028398) and symbiont LP1 of *Lyrodus pedicellatus* (AY150183). Variable regions were identified from the alignment of the 16S rRNA sequence and used to design the Q-PCR primers (16S_434F and 16S_589R, Appendix) and the TaqMan probe (16S_523, Appendix). The symbiont-specific primers Bn16S_240F and Bn16S_1253R for the 16S rRNA gene (Haygood & Davidson, 1997) were used to generate a large quantity of the specific PCR amplicon of the target for efficiency estimation and standard curve generation. Nucleotide sequences for the *bryA*, *B*, *C*, and *D* were obtained from NCBI (GenBank accession number EF032014) and aligned to determine the repeat and variable regions. The template primers, Q-PCR primers and the TaqMan probes were designed for *bryA* and *bryB* from the

variable regions. The complete sequence of *Bugula neritina* 18S rRNA gene was obtained from NCBI (GenBank accession no: AF499749). The 18S rRNA TaqMan probe, 18S Q-PCR F2 and 18S Q-PCR R2 primers were designed within the region of template primers 18S_18e F (Hillis & Dixon, 1991) and 18S_1146 R. All primers and probes used in this study are listed in the Appendix.

2.4 DNA and RNA extraction from *B. neritina* tissues

RNA and DNA were extracted sequentially from four replicates of each ovicell-bearing (OB), ovicell-free (OF) and larval (L) samples using the Trizol protocol. Briefly, tissues stored in Trizol were homogenized using a pestle, and chloroform was added to separate the upper aqueous phase and lower phenol-chloroform phase. The aqueous phase, containing RNA, was transferred to a fresh microcentrifuge tube and the RNA was precipitated by the addition of isopropanol. The RNA pellet was washed once with 75% ethanol diluted with RNase-free H₂O, and redissolved in 50 µl nuclease free water. The total RNA was further purified using the RNeasy mini kit (Qiagen, Valencia, CA). This clean up procedure was performed to ensure that the RNA samples were free of inhibitors of downstream enzymatic reactions such as cDNA synthesis. The concentration of RNA following the two protocols was measured and the purity was assessed in triplicate using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). DNA was extracted from the Trizol interphase. Ethanol (100%) was added to the interphase and phenol phase. After mixing by inversion, DNA was precipitated by centrifugation, and the pellet was washed twice in 0.1 M sodium citrate in 10% ethanol with periodic mixing for 30 min followed by centrifugation. After these two washes, the DNA pellet was finally washed in 75% ethanol and redissolved in 50 µl 8 mM NaOH, and the

pH was adjusted to 8.4 by adding 4.3 μ l of 0.1 M HEPES. The concentrations of DNA were measured in triplicate using Nanodrop spectrophotometer.

2.5 Primers and probes concentration optimization and PCR efficiency assay

All the Q-PCR primers were subjected to optimization using an Applied Biosystems 7500 Fast Real-Time PCR system to determine the primer concentration with the highest amplification efficiency. The Q-PCR reaction mixture (20 μ l) for 16S rRNA, *bryA* and *bryB* Q-PCR primers optimization contained Dynazyme II Hot Start buffer, 2.5 mM MgCl₂, 200 μ M dNTPs, 0.024 U/ μ l Dynazyme II Hot Start *Thermus brockianus* (*Tbr*) DNA Polymerase (Thermo Scientific), 150 nM - 600 nM primer, 200 nM TaqMan probe (the TaqMan probe has FAM reporter dye at 5' end and BHQ quencher dye at 3' end), 50X Rox dye (Thermo Scientific), 10 pg of template DNA and water. The following thermal cycling program was used: initial denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 30 sec, a single annealing and extension step at 60°C for 1 min and 20 sec and a final extension step at 72°C for 5 min. Each reaction was performed in duplicate. After the optimal primer concentration was determined, the optimal concentration of the TaqMan probe was assessed by Q-PCR using 100 nM and 200 nM probe concentration. The Q-PCR reaction mixture and the thermal cycling program were the same as described above. Because of the discontinuation of Dynazyme II Hot Start *Tbr* DNA Polymerase by Thermo Scientific, the DyNAmo Probe Q-PCR kit (Thermo Scientific) based on Hot Start *Tbr* DNA Polymerase was used for the 18S rRNA Q-PCR primers and probe optimization. The Q-PCR reaction mixture (20 μ l) for 18S rRNA Q-PCR primers optimization contained DyNAmo Probe Q-PCR master mix (Thermo Scientific), 150 nM - 600 nM primer, 200 nM TaqMan probe, 0.3X Rox dye, 10 pg of template DNA and water. The thermal cycling program using DyNAmo Probe Q-PCR kit consisted of initial denaturation at 95°C for 15 min, 35 cycles of denaturation at 95°C

for 15 sec, a single annealing and extension step at 60°C for 1 min and 20 sec and a final extension step at 72°C for 5 min. Each reaction was performed in duplicate.

To assess the Q-PCR primer amplification efficiency, the template primers (shown in Appendix) for 16S rRNA, 18S rRNA, *bryA* and *bryB* were used to generate PCR amplicons of target DNA, to be used as standards. The PCR amplicons were purified using QIAquick PCR purification kit and diluted in a 10-fold serial dilution. The dilutions (from 1 ng to 0.001 pg) were utilized in Q-PCR reactions to assess the amplification efficiency of the Q-PCR primers. The reaction mixture and thermal cycling program were the same as described above. Primer efficiency was calculated by using the formula $E = 10^{-1/\text{slope}}$. One hundred percent efficiency of a Q-PCR assay means that the PCR product of interest is doubling with each cycle during the logarithmic cycle of the PCR reaction. Usually, the efficiency of the assay should be above 90% and should exhibit a change of 3.3 cycles between 10-fold dilutions of sample. A standard curve was constructed by plotting the mean C_t values against the log value of template DNA concentration and each plotted points represent the linear regression line of standard curve.

2.6 Quantification of symbiont copy number per host tissues by real-time Q-PCR

DNA extracted from the three types of *B. neritina* tissues (N=3) were diluted to 180 ng/μl and 2 μl of DNA was used in Q-PCR reactions with both 16S rRNA and 18S rRNA standard curve reactions to quantify the 16S rRNA and 18S rRNA genes by absolute quantification methods (Klein, 2002). Each reaction was performed in triplicate. The copy concentration of both 16S rRNA and 18S rRNA gene per 180 ng of DNA was determined by converting the C_t value according to the standard curve. The copy number were calculated using the equation below (Whelan, *et al.*, 2003, Lee, *et al.*, 2008):

$$\text{Gene (copy)} = \frac{6.02 \times 10^{23} (\text{copies mol}^{-1}) \times \text{Q-PCR product amplicon concentration (g)}}{\text{Q-PCR product length (bp)} \times 660 (\text{gmol}^{-1}\text{bp}^{-1})}$$

The ratio of the 16S rRNA gene copy number of the symbiont bacteria and the 18S rRNA gene copy number of the host in each host tissue type was calculated.

2.7 Synthesis of cDNA

DNase digestion of RNA samples (diluted to 300 ng/ μ l) was performed using RNase-free DNase I (Fermentus) to remove DNA contamination from RNA samples. Then, 8 μ l (total 1.5 μ g RNA) of RNA was used for the cDNA synthesis with random hexamer primers and Superscript III (Invitrogen). Random hexamers are used to copy the entire length of mRNA by using the total RNA as a template for first-strand cDNA synthesis. Negative RT reactions (1.5 μ g RNA) were also performed to ensure there was no DNA contamination in the RNA.

2.8 Quantification of bryostatin gene expression by real-time Q-PCR

cDNA from each tissue type (600 ng of cDNA, N=3) was used in Q-PCR to quantify *bryA* and *bryB* gene expression. Q-PCR reactions were performed in triplicate using *bryA*, *bryB* and 16S rRNA Q-PCR primers. The relative quantification method was applied in which the relative quantity of target gene *bryA* and *bryB* were determined in OB and larval samples based on the OF samples as a calibrator that have both the target and reference gene.

3 RESULTS

3.1 DNA and RNA extraction and quantification

DNA and RNA were extracted from larvae, ovicell-free, and ovicell-bearing portions of the colonies. Purity and quantity was assessed using a Nanodrop spectrophotometer. DNA 260/280 ratios ranged from 1.6-1.8, and quantities varied from 182.8-324.5 ng/ μ l. RNA 260/280 ratios ranged from 1.9-2.3, and quantities varied from 212.0-765.3 ng/ μ l.

3.2 Primers and probes concentration optimization and PCR efficiency assay

Results from the 16S rRNA and 18S rRNA Q-PCR primer optimization experiments (Table 1) showed that the C_T value for 400 nM and 600 nM primers were very similar, and therefore, 400 nM primer concentrations was chosen for 16S rRNA and 18S rRNA gene quantification. Optimal probe concentration was determined to be 100 nM (Table 2). For the Q-PCR primer PCR efficiency assays, a standard curve was constructed by plotting the mean C_T values against the log value of template DNA concentration and each plotted points represent the linear regression line of standard curve. The Q-PCR primer amplification efficiency of both 18S rRNA and 16S rRNA Q-PCR primer were determined to be 90.63% and 92.31% respectively (Fig. 3A & 3B).

Table 1: 16S rRNA and 18S rRNA gene primer optimization by real-time Q-PCR.

Target	Primer concentration (nM)	C_T mean	C_T SD
16S rRNA	150 nM	17.83	0.24
	200 nM	17.94	0.05
	300 nM	17.80	0.17
	400 nM	17.79	0.08
	600 nM	17.76	0.02
18S rRNA	150 nM	20.05	0.17
	200 nM	19.64	0.10
	300 nM	19.35	0.12
	400 nM	19.00	0.14
	600 nM	19.57	0.12

Table 2: 16S rRNA and 18S rRNA gene TaqMan probe optimization using 100 nM and 200 nM probe concentration.

Target	Probe concentration (nM)	C_T Mean	C_T SD
16S rRNA	100 nM	16.07	0.01
	200 nM	15.92	0.20
18S rRNA	100 nM	19.31	0.01
	200 nM	19.47	0.05

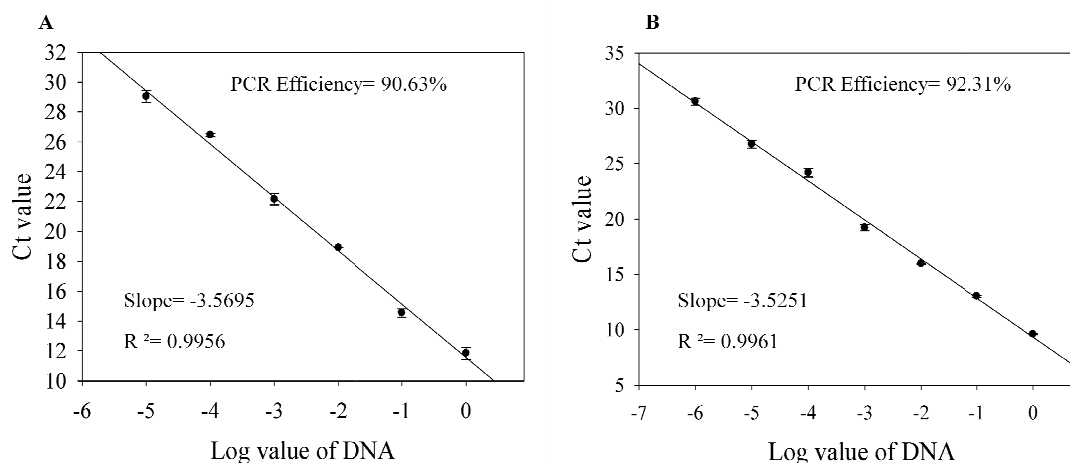


Figure 3: PCR efficiency of 18S rRNA (A) and 16S rRNA (B) Q-PCR primers.

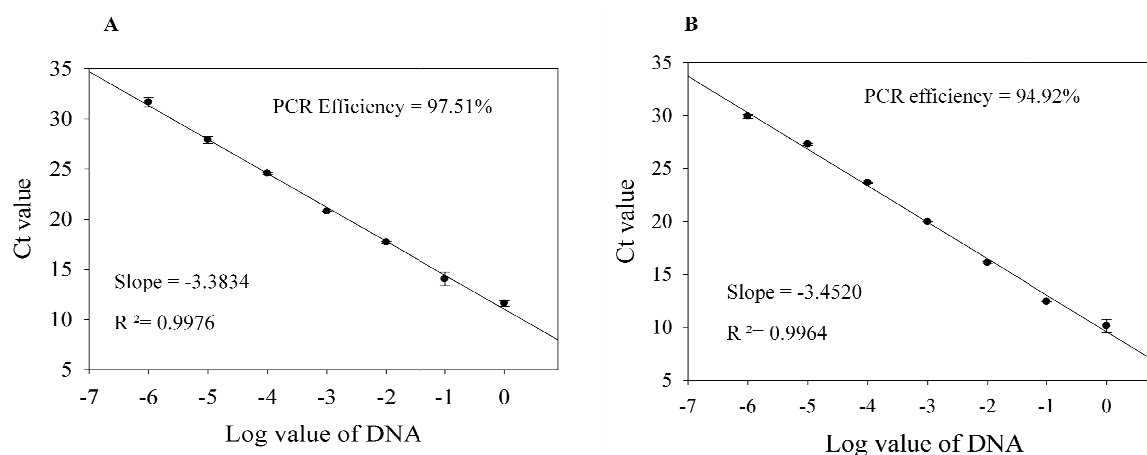
The *bryA* and *bryB* Q-PCR primer optimization results (Table 3) showed that the lowest C_t value with the least standard deviation occurred with 150 nM primer concentrations, which was used for the subsequent *bryA* and *bryB* TaqMan probe optimization experiments (Table 4). The *bryA* and *bryB* probe optimization test results (Table 4) demonstrated that the 200 nM *bryA* probe concentration resulted in the lowest C_t value and the C_t values for 100 nM and 200 nM *bryB* probe were similar. The *bryA* template primer sets *bryA*_2061F and *bryA*_2926R were used for the *bryA* PCR amplicon standard preparation. *bryB*_15707F and *bryB*_16523R were used for *bryB* PCR amplicon standard preparation. A 10-fold serial dilution (from 1 ng to 0.001 pg) of purified *bryB* and *bryA* PCR amplicons were used to generate a standard curve (Fig. 4A & 4B) to assess the amplification efficiency of the Q-PCR primers (using 150 nM of forward and reverse *bryA* Q-PCR primers, 200 nM *bryA* TaqMan probe and 150 nM of forward and reverse *bryB* Q-PCR primers, 100 nM *bryB* TaqMan probe). The *bryA* and *bryB* Q-PCR primer amplification efficiency was calculated in same way described above for 16S rRNA and 18S rRNA. The amplification efficiency for *bryB* was 97.51% and for *bryA* was 94.92% (Fig. 4A & 4B).

Table 3: *bryA* and *bryB* Q-PCR primer optimization by real-time Q-PCR.

Target	Primer Concentration (nM)	C _T Mean	C _T SD
<i>bryA</i>	150 nM	12.86	0.13
	200 nM	12.86	0.09
	300 nM	12.64	0.19
	400 nM	12.97	0.30
	600 nM	12.70	0.10
<i>bryB</i>	150 nM	14.16	0.11
	200 nM	14.19	0.08
	300 nM	14.36	0.23
	400 nM	14.20	0.02
	600 nM	14.07	0.05

Table 4: *bryA* and *bryB* TaqMan probe optimization using 100 nM and 200 nM probe concentration.

Target	Probe Concentration (nM)	C _T Mean	C _T SD
<i>bryA</i>	100 nM	13.68	0.15
	200 nM	12.53	0.10
<i>bryB</i>	100 nM	13.70	0.02
	200 nM	13.51	0.04

**Figure 4:** PCR efficiency of *bryB* (A) and *bryA* (B) Q-PCR primers.

3.3 Quantification of symbiont copy number per host tissues by real-time Q-PCR

To quantify symbiont 16S rRNA gene copy number and host 18S rRNA gene copy number by absolute quantification methods (Klein, 2002), 2 µl of metagenomic DNA (180 ng/µl) from three types of *B. neritina* tissues (N=3) was used in Q-PCR along with both 16S rRNA and 18S rRNA gene standard curve reactions. Each reaction was performed in triplicate. The copy concentration of both 16S rRNA and 18S rRNA gene per 180 ng of DNA was determined by interpolating the C_T value to the standard curve (the r² value of the linear regression for the 16S rRNA standard curve is 0.999 and, for 18S rRNA standard curve, is 0.995). The copy number for each gene was calculated using the equation described in the Materials and Methods above. The ratio of 16S rRNA gene copy number of the symbiont bacteria and 18S rRNA gene copy number of the host were calculated (Table 5) to approximate the density of symbiotic bacteria in each type of host tissue. The statistical test result (Table 6) of ratio of samples (determined by Independent T-Test) showed that there are no significant differences in the ratio among tissues. One replicate of each OB and OF tissue was excluded from the statistical analysis due to high standard deviation of C_T values.

Table 5: Quantification of 16S rRNA and 18S rRNA gene by Q-PCR.

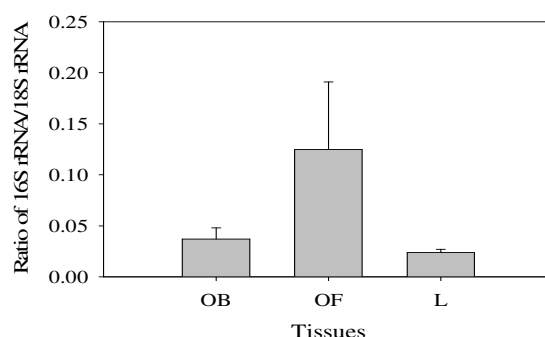
Sample	*Ratio (16S rRNA/18S rRNA)	#Average Ratio	#Std. Deviation
OB2	0.044	0.037	0.011
OB3	0.029		
OF1	0.171	0.125	0.066
OF2	0.078		
L1	0.023	0.024	0.003
L2	0.022		
L3	0.028		

* represented as the average ratio of three technical replicates for each biological replicate.

represented as average ratio and standard deviation of ratio of biological replicates for each tissue type.

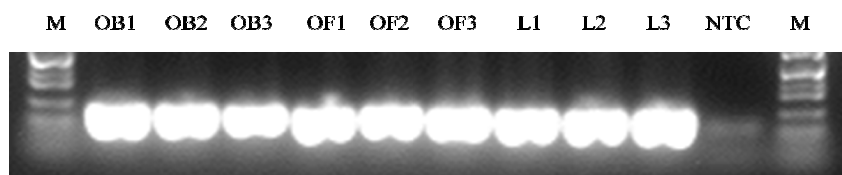
Table 6: Statistical test results of 16S rRNA/18S rRNA gene ratio.

Statistical test	Sig. (2-tailed)	Result
Independent T-Test	0.340	16S rRNA/18S rRNA ratio of larvae and OB are not significantly different
	0.303	16S rRNA/18S rRNA ratio of OB and OF are not significantly different
	0.276	16S rRNA/18S rRNA ratio of larvae and OF are not significantly different

**Figure 5:** Average ratio of 16S rRNA/18S rRNA gene of *B. neritina* tissues.

3.4 PCR amplification of cDNA

To confirm cDNA synthesis, the first-strand cDNA obtained in the synthesis reaction along with -RT control reactions were PCR amplified with 18S rRNA Q-PCR primers and the products were analyzed using agarose gel electrophoresis. The 18S rRNA gene PCR product was observed for +RT reactions demonstrating that cDNA synthesis reaction was successful (Fig. 6) whereas no 18S rRNA gene PCR product was observed for -RT reactions (negative control) indicating that the RNA was not contaminated with genomic DNA (data not shown).

**Figure 6:** Agarose gel electrophoresis of 18S rRNA gene PCR products of cDNA samples. Samples OB1, OB2, OB3, OF1, OF2, OF3, L1, L2, L3, lane M: HiLo DNA ladder and lane NTC: No template control.

3.5 Quantification of bryostatin gene expression by real-time Q-PCR

The *bryA* and *bryB* gene expression quantification in three types of tissues of *B. neritina* was performed using Q-PCR, and their expression levels were compared relative to expression of the 16S rRNA gene. The C_T value of larval cDNA samples for *bryB* were undetermined and the C_T value for *bryA* ranged from 33.2-34.8, suggesting that the quantity of *bryA* and *bryB* target gene expression was very low in larval cDNA. The fold change in *bryA* and *bryB* gene expression and statistical significance of relative gene expression was determined using Relative Expression Software Tool (REST 2009). The expression of target genes *bryA* and *bryB* was normalized to the reference gene 16S rRNA. The relative expression ratio of *bryA* and *bryB* was calculated using the mathematical algorithms with the following equation (Pfaffl, *et al.*, 2002) where E = Real-Time PCR efficiency, ΔCP = the crossing point difference of target sample versus a control sample.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}} (\text{Mean control} - \text{Mean sample})}}{(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}} (\text{Mean control} - \text{Mean sample})}}$$

OB and larval samples were used as target samples and OF samples were used as control samples. REST analysis demonstrated that *bryA* is significantly (p=0.001) down-regulated in larval tissue (N=3) in comparison to the OF control group (Table 7). *bryA* and *bryB* gene expression is not significantly different in OB tissue (N=3) in comparison to OF control group (p=0.730, and p=0.749 respectively) (Table 7).

Table 7: Relative gene expression using Relative Expression Software Tool (REST 2009).

Gene	Reaction efficiency	Expression Ratio		Std. error	95% C.I.	P value	Result
<i>bryA</i>	94.92	OB	0.851	0.475-1.785	0.250-2.482	0.730	Not different from control group
		L	0.064	0.032-0.107	0.031-0.117	0.001	Down regulated
16S rRNA	92.31	OB	1				
		L	1				
<i>bryB</i>	97.51	OB	1.077	0.675-1.946	0.523-2.391	0.749	Not different from control group

4 DISCUSSION

Symbiont-produced natural products play an important role in host survival by providing them protection from predators, competitors, as well as pathogens (Pawlik, 1993, Hay, 1996, Amsler, *et al.*, 2001, McClintock & Baker, 2001, Engel, *et al.*, 2002). Variable concentrations of defensive natural products within an organism have been shown to occur in some sponges and in gorgonians (Harvell, *et al.*, 1996, Lindquist & Hay, 1996, Uriz, *et al.*, 1996). It is hypothesized that these compounds, which are often defensive in nature are accumulated in areas that are most vulnerable. It can be hypothesized that organisms that produce the natural products *de novo* have evolved mechanisms for transporting the compounds to the most vulnerable regions, although specific mechanisms for this have not been elucidated. For secondary metabolites produced by a symbiotic partner, it seems reasonable to suspect that this may have occurred over their co-evolutionary history as well. In the marine bryozoan, *Bugula neritina*, the large, soft-bodied larvae are released during the day and are extremely susceptible to predation. Previous research has shown that *B. neritina* larvae have 10X more defensive bryostatins than adult colonies (Lopanik, *et al.*, 2006), despite the fact that the bryostatin-producing symbiont is present in all

life stages of the host. The primary goal of this research was to examine potential mechanisms for the concentration of bryostatins on developing embryos and larvae.

Variation in symbiont-produced bryostatin concentrations within *B. neritina* colonies could be the result of higher titers of "*E. sertula*" in zooids that are brooding embryos compared to zooids that are not, to increased production of bryostatins by "*E. sertula*" in reproductive zooids, or a combination of both. Alternatively, both symbiont distribution and bryostatin production could be uniform within all portions of the colony, and bryostatins are transmitted to ovicells via funicular cords. In order to assess the density of "*E. sertula*" cells in each host tissue type, the ratio of 16S rRNA gene copy number to 18S rRNA gene copy number was calculated. The average ratio of 16S rRNA to 18S rRNA gene is slightly higher in OF and OB samples than in larval samples, although this difference is not statistically different, suggesting that symbiont cells are not accumulated in ovicells that are brooding a larva. This result obtained refutes the original hypothesis, and suggests that similar numbers of bacteria are hosted by OB and OF tissue.

Another possible scenario resulting in higher bryostatin levels in ovicell-bearing zooids is that "*E. sertula*" cells associated with ovicells up-regulate bryostatin biosynthesis, perhaps due to a signal produced by the host. To assess this hypothesis, expression of bryostatin biosynthetic genes *bryA* and *bryB* in three types of tissue was compared to expression of the 16S rRNA gene. The C_T value for 16S rRNA of OB and OF samples were similar ($p=0.09$, ANOVA, although this is close to 0.05) demonstrating that the expression of 16S rRNA is invariant in each type of tissue; this suggests that the expression of the 16S rRNA gene is appropriate for normalization of *bry* gene expression in this study. The C_T value for *bryB* was undetermined in all larval cDNA sample replicates, while the C_T value for *bryA* gene was higher (ranges 33.2-34.8) in all larval

cDNA samples. This result suggests that *bryA* and *bryB* gene expression in larvae may occur at very low levels, and relative gene expression analysis demonstrates that *bryA* gene is significantly down regulated ($p=0.001$) in larval samples compare to the ovicell-free control samples (Table 7). Experiments have suggested that bryostatins are localized on the surface of larvae during embryogenesis (Lopanik, *et al.*, 2004a, Sharp, *et al.*, 2007). The coating of bryostatins lingers onto the developing larvae until the release of matured larvae from ovicell and the formation of first feeding zooid (Sharp, *et al.*, 2007). Bryostatin signal was not detected in the early metamorphic stages of larvae suggesting that bryostatin synthesis has not started in this early stage. However, the results of another study of Davidson and coworkers (2001) used FISH to demonstrate expression of a β -ketoacyl synthase (KS) gene fragment of *bry* in the pallial sinus of larvae. In this study, the down regulation of *bryA* gene expression in larval tissue compared to adult OF tissue suggests that low levels of bryostatin biosynthesis occur in larvae. Relative gene expression analysis reveals that both *bryA* and *bryB* gene expression is not significantly different ($p>0.05$) in OB samples compare to the control sample OF (Table 7) which refutes the hypothesis that bryostatin biosynthetic gene is up-regulated in OB tissue. The combined results from the two experiments performed in this study suggests that the most likely scenario is that bryostatin is produced at uniform levels throughout the host colony, but transported to the developing embryo during embryogenesis via the funicular cords. The findings of this study suggest that the host play a role in regulating bryostatin transport within a colony and to the developing larvae.

5 CONCLUSION

The objective of this study was to investigate the role that symbiont density and differential bryostatin biosynthetic gene expression play in the irregular distribution of bryostatins in the *B. neritina* colony. The titer of symbiotic bacteria and relative bryostatin biosynthetic gene expression within three types of *B. neritina* tissue was quantified using TaqMan probe based Q-PCR in order to identify the potential mechanism that results in varied levels of bryostatins in larvae, in ovicell-bearing and ovicell-free zooids. Neither the number of bryostatin-producing symbiotic bacteria, nor bryostatin gene expression was significantly different in adult ovicell-bearing and ovicell-free tissues. This suggests "*E. sertula*" uniformly distributed within the *B. neritina* colony produces a constant level of bryostatins, which are translocated to developing larvae. It is possible that *B. neritina* is involved in regulating the transfer of bryostatin to the developing larvae via the funicular cords. The outcome of this study sheds light on the mechanisms of localization of natural products in host tissue and expands understanding of host-symbiont interactions in a marine defensive symbiosis.

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7 APPENDIX

Primers and TaqMan® probes used in PCRs and Q-PCR.

Name	Sequence (5'-3')	Reference
bryA_2739F	CATTTGCCACGAGTTTCTCA	This study
bryA_2877R	CCTGTCACAAACATCAACGG	This study
bry A_2061F	GTATGGACATATTCCTTATC	This study
bry A_2926R	GTATCAACTTTTAATACGGT	This study
bryA_2802 probe	AAGGTCTATTCAGGAAATAT	This study
bryB_16213F	GCCTTAGAATTAGATCTGGACGA	This study
bryB_16301R	AGTGGCTATTGATTTTTCGTATCC	This study
bryB_15707F	TTCATGCTAGTCGAGTGTTGC	This study
bryB_16523R	TTCAAATAATTTTCTGCCTTATCAA	This study
bryB_16276probe	TTGGATTTCGATAAATGGAGTTA	This study
18S_18e F	CTGGTTGATCCTGCCAGT	Hillis and Dixon, 1991
18S_1146 R	TTAAGTTTCAGCTTTGCAACCA	Unknown source
18S Q-PCR F2	CCGGCGACGCCTGCACTGAG	This study
18S Q-PCR R2	CGTGCCTGCTGCCTTCCTTG	This study
18s_341 probe	TTGACGGATAACAGAGAAT	This study
16S_434F	GAGGAGGAAAGGTTGACGAA	This study
16S_589R	CCGGGGATTTCACATCTG	This study
Bn16S_240F	TGCTATTTGATGAGCCCGCGTT	Haygood and Davidson, 1997
Bn16S_1253R	CATCGCTGCTTCGCAACCC	Haygood and Davidson, 1997
16S_523 probe	ATACGGAGGGTGCGAGC	This study